A MODIFIED ADHESIVE SYSTEM FOR USE IN TREATMENT OF DENTIN HYPERSENSITIVITY

by

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DEDICATION

I dedicate this thesis to my mom, Jamilah, and my dad, Mushabbab, who have worked so hard to make me the person I am today. I owe everything to you, and I hope I make you proud.

I also dedicate this thesis to the people I love the most, my siblings, Kholoud, Mohammad, Asma and Mashael.

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INTRODUCTION

The treatment of dentin hypersensitivity (DH) can be challenging and unpredictable despite the variety of treatment options available. DH may have similar clinical symptoms to other dental conditions such as caries, defective restorations, cracked-tooth syndrome and pulpitis. Therefore, all possible causes should be excluded before making a diagnosis of DH. Many treatment modalities are available for management of dentin hypersensitivity; nevertheless, most of them are not satisfactory in the long term and re-treatment may be required.

DH has been defined as a condition characterized by short, sharp pain arising from exposed dentin in response to stimuli, typically thermal, evaporative, tactile, osmotic or chemical, and which cannot be ascribed to any other form of dental defect or pathology.[1] In 1966 Brännström presented evidence to support the hydrodynamic theory,[2] which was proposed earlier in 1900,[3] to explain the pain associated with DH. This theory states that when a dentin surface is exposed, certain stimuli can trigger a rapid outward movement of the fluid inside the dentinal tubules surrounding the odontoblastic processes that extend inside the tubules, which in turn stimulates the A-delta nerve fibers around the odontoblasts, initiating a sharp pain sensation.[4]

Two other theories have been proposed to explain pain associated with DH; the direct innervation theory and the odontoblastic transducer theory. The direct innervation theory was the very first theory put forward to explain DH. It states that dentin is innervated and pain is elicited by direct stimulation of nerve endings inside the dentinal tubules. The odontoblastic transducer theory came later to explain that odontoblasts,

coming from a neural crest origin, act as the initial sensory receptors that connect with nerve fibers inside the pulp to transmit pain.[5] However, evidence fails to support these two theories, and the hydrodynamic theory remains as the most widely accepted theory to explain pain associated with DH.[6-8]

Based on the hydrodynamic theory, a logical treatment strategy for obtaining pain relief is occluding patent dentinal tubules to prevent stimuli from triggering movement of the dentinal fluid that would stimulate nerve fibers. Another approach to treat DH aims to block neural conduction of pain. This is achieved by utilizing potassium salts, most popularly potassium nitrate, or others such as potassium chloride or potassium citrate, which penetrate the dentinal tubules all the way to the pulp and prevent repolarization of pulpal nerves, inhibiting their ability to transmit pain signals to the brain.[9-11] While potassium salts have the ability to block neural pain transmission, the majority of the other agents used such as calcium, fluoride, oxalate, and strontium salts act by occluding the dentinal tubules. Some agents are able to deposit or stimulate deposition of a plugging material inside the tubules, while others, such as resin-based dentin bonding agents or restorative materials, mainly cover the dentin surface and form a bonded seal. Various challenges in the oral environment such as salivary flow, acids from bacterial or dietary sources and mechanical forces can cause dissolution of the occlusive material, resulting in recurrence of pain. Precipitation of occlusive material deeper in the dentinal tubules seems to make it more resistant to dissolution, resulting in a longer-lasting pain relief.[12] An ideal treatment agent for DH would be one that is easily applied, has a rapid, permanent effect, does not cause pulpal irritation and does not stain teeth.[13]

Natural mechanisms for dentinal tubule occlusion and reducing DH exist with saliva being a source of calcium and phosphate needed for mineral precipitation and natural tubule occlusion.[14,15] Salivary glycoproteins can also aggregate to assist in plugging open dentinal tubules. These mineral precipitation and protein aggregation processes occur more readily in an alkaline pH.[16] In general, these natural mechanisms are usually slow and dependent on the quantity and quality of saliva. In individuals where the oral environment is continuously acidic, the mineral precipitation and salivary protein aggregation are not likely to be effective in reducing DH.[14,16]

Extensive studies of the salivary mechanisms for natural tubule occlusion have resulted in the introduction of a salivary-based product for treatment of DH.[14] This product was an arginine-bicarbonate-calcium carbonate complex used as a prophylaxis paste. Arginine is a basic amino acid that exists in saliva in its free form,[17] as well as in the salivary peptides and proteins, which are considered its main source.[18] Arginine has a well-established effectiveness in raising the surrounding pH by being metabolized by alkali-producing oral bacteria.[19] This results in a favorable environment for mineralization of tooth structure especially when supported by the availability of calcium. Because of the calcium carbonate, this product forms a plug with low solubility in saliva that adheres to the tooth surface.[14] In the event of an acid attack, any calcium carbonate that is dissolved will lead to the release of arginine that can help in counteracting the acidity.[14]

To help in the selection of the most suitable treatment option for a particular case of DH, it has been recommended to use over-the-counter (OTC) desensitizing products for mild DH; in-office applied varnishes and other “reversible” or “non-invasive”

desensitizing agents for moderate DH followed by home-use of OTC desensitizing products; and “semi-invasive” treatment modalities for severe DH, including the use of bonding agents, composite resin or glass ionomer restorations to provide a long-term sealing of the dentinal tubules.[20,21]

The use of adhesive resins for blocking the tubules was suggested as early as 1979.[22] Brännström et al. recommended resin impregnation as an alternative to other methods for an immediate and permanent relief of pain associated with DH.[22] Modification of dentin bonding agents has been attempted for various purposes in dentistry. The literature shows different active components, such as anti-bacterial and bio-active agents, being incorporated into adhesive systems using different strategies in order to improve the performance of these resins in a specific area. In 2013 Bottino et al. reported on modifying an adhesive resin using halloysite clay nanotubes (HNT), which was the first report of using HNT to modify the adhesive resin of a commercial unfilled bonding agent.[23] Halloysite is a naturally occurring clay mineral in the form of a bi-layer aluminosilicate sheet that exists as hollow nanotubes with an external diameter of 50 nm to 70 nm, lumen diameter of 10 nm to 15 nm, and a length of about 1 μm.[24] These nanotubes are biocompatible and can be used as chemical carriers loaded up to 10 wt.% to 30 wt.%.[25] An interesting feature of HNT is that the inner layer is alumina and the outer layer is silica, giving different chemical properties for these two inner and outer layers.[25] The positively charged inner lumen and negatively charged outer surface of the HNT allow for selective loading of negative molecules on the inside and positive ones on the outside of the nanotubes. An important advantage of using HNT for drug delivery is the potential for sustained, slow release of therapeutic components over hours, days,

weeks or even months for a longer-lasting effect, along with the mechanical reinforcement that these nanotubes can provide.[24-26]

The aim of the present study was to modify the dentin primer and adhesive of a commercial adhesive system with arginine and calcium carbonate-encapsulated nanotubes; to evaluate the modified system's cytocompatibility, viscosity and efficacy in reducing dentin permeability, and to obtain insights about the system's possible use as a treatment for dentin hypersensitivity

NULL HYPOTHESES

- There will be no difference between the modified and non-modified adhesive systems regarding cytocompatibility.
- There will be no difference between the modified and non-modified adhesive systems regarding viscosity.
- There will be no difference between any of the experimental groups and the control groups in the ability to reduce dentin permeability after cycling.

ALTERNATIVE HYPOTHESES

- There will be a difference between the modified and non-modified adhesive systems regarding cytocompatibility.
- There will be a difference between the modified and non-modified adhesive systems regarding viscosity.
- There will be a difference between the experimental and control groups in the ability to reduce dentin permeability after cycling.

REVIEW OF LITERATURE

The literature shows great variations in the reported prevalence of DH ranging from 1.34 percent[27] to 92 percent.[28] This may be attributed to variations in the populations studied (healthy vs. periodontally involved dentition), practice settings (general dental practice vs. periodontal practice), recruitment methods (self- vs. clinician-based recruitment), and methods for diagnosing DH (patient questionnaire vs. clinical examination). A systematic review and meta-analysis conducted in 2018 in an effort to provide an estimate of the prevalence of DH reported that the best estimate was 11.5 percent, and the average of all included studies was 33.5 percent.[29]

Dentin hypersensitivity is usually found in teeth with anatomical characteristics that make them more susceptible to being hypersensitive. Normally, dentinal tubules have diameters ranging from about 3 μm at the pulp, to about 0.5 μm peripherally near the dentin-enamel junction (DEJ).[30] Consequently, the density of tubules decreases from about 60,000/mm^2 near the pulp to 10,000/mm^2 at the DEJ.[31] It has been demonstrated that teeth with hypersensitive dentin have a greater number of dentinal tubules per unit area (approximately 8 times) and wider diameters of these tubules (approximately 2 times) compared with non-sensitive teeth.[32] Also, hypersensitive areas of dentin were found to have more open tubules as opposed to more obturated tubules in the non-sensitive areas.[33] The radius of dentinal tubules was found to be the most important factor influencing fluid flow inside these tubules.[34] The dentinal fluid flow rate is directly proportional to the fourth power of the dentinal tubule radius. This means that small reductions in the radius can result in much greater reductions in the fluid flow.[34]

Extensive laboratory studies of the structure of dentin and how it relates to its permeability have contributed to a better understanding of dentin hypersensitivity. In 1974 Outhwaite et al. demonstrated measuring the hydraulic conductance of slices of coronal dentin under a source of hydrostatic pressure to evaluate their permeability.[35] The hydraulic conductance is the ease with which fluid can move across a unit surface area under a unit pressure per unit time.[36] This method became one of the most widely implemented for evaluating dentin permeability. Another approach to evaluate permeability is through imaging to visualize occlusion of dentinal tubules. Scanning electron microscopy (SEM) studies are widely used for that purpose. In clinical studies, dentin hypersensitivity is usually evaluated by scoring the pain level most commonly on a visual analogue scale.

Clinical studies have demonstrated the effectiveness of desensitizing products containing arginine. A study has shown that a single professional application of a paste containing 8.0-percent arginine and calcium carbonate showed an instant sensitivity relief that lasted for 28 days and was significantly better compared with a control pumice prophylaxis paste.[37] Two eight-week clinical trials demonstrated the superior efficacy of a toothpaste containing 8.0-percent arginine, calcium carbonate, and 1450-ppm fluoride compared with a commercial toothpaste containing 2.0-percent potassium ion and 1450-ppm fluoride in reducing DH.[38,39]

Whether specifically developed for treating hypersensitive dentin or not, various forms of dentin bonding agents have been used for treatment of DH with success. Seal&Protect® by Dentsply Sirona is a self-etching UDMA-based resin material that was developed specifically for sealing dentinal tubules.[40] Some of its important components

are nanofillers for reinforcement and increased abrasion resistance and Triclosan for an anti-bacterial effect.[40] BisBlock is an oxalate dentin desensitizer that acts by forming calcium-oxalate crystals within the tubules.[41] The product kit contains a phosphoric acid etchant to be used before the oxalate agent, and a dentin bonding agent to be used after the oxalate agent to provide a seal over the crystals that are formed within the tubules and improve longevity.[41]

Several studies report the effects of incorporating HNT into adhesive resins on their properties.[23, 42-46] Incorporation of up to 20 wt.% HNT into the adhesive resin of etch-and-rinse three-step[23] and two-step[42] adhesive systems can enhance the shear bond strength to dentin without jeopardizing other important material properties such as microhardness and degree of conversion.[23] HNT were shown to be non-toxic to cells and to exhibit high cell biocompatibility and a very low level of cytotoxicity.[47] Incorporating up to 20 wt% HNT into an adhesive resin did not increase its cytotoxicity to human dental pulp stem cells compared with a commercially available adhesive resin without HNT.[43] The same was found when chlorhexidine [44] and doxycycline [45] were encapsulated into HNT and used to modify the adhesive resins.

After the promising potential of adhesives modified with HNT were made clear, it was recommended to explore the potential of modifying adhesive primers with HNT and how that would affect the durability of the resin-dentin bond.[23] A recent study investigated the effect of incorporating chlorhexidine-encapsulated-HNT into the primer, as well as the adhesive, of a commercial three-step adhesive system on bond strength to dentin.[48] The results showed that the group with the modified primer and non-modified adhesive had significantly greater micro-tensile bond strength to dentin after 6 months of

aging than the group with the modified adhesive and non-modified primer. This sheds light on an area of research that would be interesting to explore.

In summary, acidity in the oral environment seems to be a major challenge that hinders a long-term dentin desensitizing effect regardless of the modality of treatment used. The use of resin bonding may have a mechanical advantage in occluding the dentinal tubules for a longer time. Modifying an adhesive system with arginine and calcium carbonate-encapsulated nanotubes may result in a promising, unique agent for treatment of DH that combines mechanical and therapeutic benefits.

MATERIALS AND METHODS

This laboratory study was reviewed and approved by the local Institutional Review Board of Indiana University under the IRB protocol number 181096811. The study included two phases:

PHASE I: Synthesis and characterization of the experimental primer and adhesive, including:

- Cytocompatibility testing.
- Viscosity testing.

PHASE II: Dentin permeability testing, including:

- Baseline dentin permeability testing.
- Treatment application.
- Post-treatment dentin permeability testing.
- Erosion-abrasion-remineralization cycling.
- Post-cycling dentin permeability testing.

SYNTHESIS OF THE EXPERIMENTAL ARGININE-CALCIUM CARBONATE-HNT PRIMER AND ADHESIVE

Synthesis of Arginine and Calcium Carbonate-encapsulated HNT Powder

Arginine (Arginine, free base, MPI) and calcium carbonate ($CaCO_3$) (ACS reagent, ACROS organics) were added in a tube containing ethanol. The tube was placed on a vortex mixer (MaxiMix Plus ^{TM}, Thermolyne, Dubuque, IA, USA) and then transferred to an end-to-end mixer (Roto-Rack Tube Rotator, Fisher Scientific, USA).

After that, HNTs (Dragonite 1415JM, Applied Minerals, New York, NY, USA) were added to the tube and it was placed on the vortex mixer and then sonicated in an ultrasonic machine (L&R Ultrasonic Cleaning System 2014, Kearny, NJ, USA). The tube was then placed in a vacuum machine (VWR vacuum oven, Radnor, PA, USA) under 25 in Hg and then in the end-to-end mixer overnight. The next day, the tube was placed in the vacuum machine and then in a centrifuge machine (Beckman GPR Centrifuge, Indianapolis, IN, USA) at 800 rpm in order to precipitate the powder at the bottom of the tube (Figure 1.a). After that, the liquid that surfaced on top of the powder was removed and the tube was placed in a 37°C incubator (HeraTherm oven, Thermo Scientific, Waltham, MA, USA) and left for one week.

Arginine-$CaCO_3$-HNT Powder Characterization

The morphology of the HNT powder was examined before and after encapsulation with arginine and $CaCO_3$ under transmission electron microscopy (TEM) (Tecnai Spirit TEM, Thermo Fisher Scientific, Hillsboro, OR, USA) (Figure 2).

Incorporation of the Arginine-$CaCO_3$-HNT Powder into a Dentin Primer and Adhesive

Two experimental agents were prepared, an arginine-$CaCO_3$-HNT-modified dentin primer and an arginine-$CaCO_3$-HNT-modified dentin adhesive. The primer and adhesive from the Adper ™ Scotchbond ™ Multi-Purpose Plus (SBMP) adhesive system (3M ESPE, St. Paul, MN, USA) were utilized and modified by incorporating the arginine-$CaCO_3$-HNT powder at 15 wt%. Agents were prepared immediately before use. In a dark controlled-lighting room, SBMP primer was pipetted into an amber jar and the arginine-$CaCO_3$-HNT powder was added. A mechanical mixer (Scilogex D160

Homogenizer, Rocky Hill, CT, USA) was then used to homogenize the liquid and powder at 8000 rpm. After that, the jar was transferred to the vortex mixer and mixed and then immediately used. The same process was followed to prepare the arginine-$CaCO_3$-HNT-modified adhesive (Figure 1.b, c and d).

CHARACTERIZATION OF THE EXPERIMENTAL PRIMER AND ADHESIVE

Cytocompatibility

To evaluate cytocompatibility of the experimental primer and adhesive and compare it to the control, commercial primer and adhesive, disc-shaped specimens (6 mm diameter × 0.75 mm thickness) of four different combinations of primers and adhesives were prepared (n=4/group); SBMP: non-modified primer and adhesive, HNT-PR: modified primer and non-modified adhesive, HNT-ADH: non-modified primer and modified adhesive, HNT-PR+ADH: modified primer and adhesive (Figure 3). On a glass slab, a plastic mold of a disc shape was placed with a transparent film under, and equal amounts of primer and adhesive were pipetted into the mold, another piece of transparent film was placed over the filled mold that was then pressed gently on top with another glass slab and light-cured for 10 seconds on each side using a light-emitting diode curing light (Bluephase® Style, Ivoclar Vivadent, Amherst, NY, USA) with an output intensity of 1300 mW/cm^2 as verified with a radiometer (Cure Rite Visible Curing Light Meter, Dentsply Caulk, USA). Specimens were kept in a 37°C incubator for 24 hours. After that, specimens were disinfected by exposure to ultraviolet (UV) light in a cell culture hood for 30 minutes on each side (Figure 4). Next, specimens were individually incubated in serum-free Dulbecco's Modified Eagle Medium with 1 g/L glucose, L-glutamine, and

sodium pyruvate (DMEM) (CORNING® cellgro®, Corning, NY, USA) at a volume ratio of 1:4 medium to specimen by adding 5 ml of the DMEM to each disc in a 15 ml tube (Figure 5). Tubes were then placed in an incubator shaker (C25 Incubator Shaker, New Brunswick Scientific, Edison, NJ, USA) at 100 rpm and 37°C for 24 hours. After 24 hours, aliquots were collected by separating the media from the discs into new 15 ml tubes. Tubes containing the collected aliquots were stored in a -20°C freezer.

To evaluate the effects of the tested materials on human gingival fibroblast (HGF) cells, three dishes of sub-confluent HGF cells were used and 5 ml of 0.25-percent Trypsin-EDTA solution (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) was added to each dish. When cell detachment was verified under light microscopy, 5 ml of serum-plus DMEM was added to each dish to neutralize the effects of Trypsin-EDTA. Tubes were then centrifuged (IEC Medlite Microcentrifuge, Thermo Scientific, Waltham, MA, USA) at 300 × g for 10 minutes to precipitate the cells. The liquid on top of cells was removed and serum-plus DMEM was added. Cells were counted using a hemacytometer (Hausser Scientific, Horsham, PA, USA). The diluted cell suspension was seeded in four 24-well plates with a cell density of ≅ 20,000 cells per well. Plates were incubated in a humidified incubator at 37°C and 5.0-percent CO_2 for 24 hours. After that, media in the wells were removed and replaced with five different concentrations of the collected aliquots diluted with serum-free DMEM as follows; 100%, 50%, 25% 12.5% and 0% (control) aliquots. Each well received a total of 500 µl of solution based on the corresponding concentration. Plates were placed back in the humidified incubator at 37°C and 5.0-percent CO_2 for 72 hours. Images of the cells during incubation were obtained under light microscopy (Nikon Digital Sight DS-Fi1 camera with NIS-Elements

imaging software version 4.0) to visualize changes (Figure 6). At the end of the 72-hour incubation period, colorimetric analyses of cell death and cell proliferation utilizing Lactate Dehydrogenase Activity (LDH) and Water-Soluble Tetrazolium salts (WST-1) assays, respectively, were conducted.

LDH Assay

This assay quantifies cell death by detecting lactate dehydrogenase activity released from lysed cells into the cell supernatant. The cell supernatants from all wells in the 24-well plates were transferred into 2.0- ml microcentrifuge tubes. After that, a mixture of the lysis solution (Cytotoxicity Detection Kit^{PLUS} (LDH) bottle (3), Roche Diagnostics, Indianapolis, IN, USA) diluted with serum-free DMEM at 1:1 ratio was prepared and 500 µl of it was added to wells of the 24-well plates that contained 0.0-percent aliquot concentration and plates were placed back in the incubator. When cell lysis in these wells was verified under light microscopy, a 100 µl of this reacted lysis solution was pipetted into wells of the 96-well plates. Each 96-well plate contained five columns of wells containing the five aliquots concentrations of 100%, 50%, 25% 12.5% and 0%, a column containing the reacted lysis solution that was used as a high control (maximum cell lysis and LDH release) and a column containing serum-free DMEM with no cell interaction that was used as a background control. The LDH reagent was prepared according to the manufacturer's instructions (Cytotoxicity Detection Kit^{PLUS} (LDH), Roche Diagnostics, Indianapolis, IN, USA) and a 100 µl of this mixture was added to each well in the 96-well plates (Figure 7.a). The 96-well plates were incubated in the dark at room temperature for 30 minutes and then placed in a microplate reader (Molecular Devices ThermoMax, San Jose, CA, USA) (Figure 8) and light absorbance was measured

at 490 nm. The percentage cytotoxicity was calculated using the following equation: Cytotoxicity (%) = (experimental absorbance value – background control absorbance value)/ (high control absorbance value – background control absorbance value) ×100.

WST-1 Assay

To detect metabolically active cells, a tetrazolium salt (CytoSelect™ WST-1 Cell Proliferation Assay Reagent, Cell Biolabs, San Diego, CA, USA) is added to cells, which is then cleaved by live cells into a colored formazan form that produces a color change that can be read by a spectrophotometer microplate reader. More cell proliferation will lead to more formazan formation and result in a higher signal reading.

WST-1 reagent was prepared by dilution at 1:9 WST-1 reagent to serum-free DMEM and 500 µl of this mixture was added to cells in the 24-well plates and placed back in the incubator until cell lysis was verified under light microscopy. After that, 100 µl of this reacted mixture was transferred from the 24-well plates into 96-well plates, with five columns of wells containing the five aliquots concentrations of 100%, 50%, 25% 12.5% and 0% and a sixth column containing 100 µl of the prepared WST-1 reagent (not interacted with cells) to be used as a background control (Figure 7.b). The wells containing 0.0-percent aliquots concentration were used as the high control (maximum cell proliferation). The 96-well plates were then placed in the microplate reader and light absorbance was measured at 450 nm. The percentage cell viability was calculated according to the following equation: Cell viability (%) = (experimental absorbance value – background control absorbance value)/ (high control absorbance value – background control absorbance value) ×100.

Viscosity

To determine viscosity of the experimental primer and adhesive and compare it to that of commercial primer and adhesive, a cup-and-cone rotational viscometer (DV-II + Viscometer, Brookfield AMETEK®, Middleborough, MA, USA) with a cone-shaped spindle (CPA-52Z) was used (Figure 9). The viscometer was calibrated following the manufacturer's instructions. According to the requirement of the spindle that was used, 0.5 ml of each tested material was placed in the sample cup and viscosity was measured for three samples of each material. At first, different rpm values were tried for primers and adhesives to figure out a suitable rpm that gives the most stable viscosity readings. The reported viscosity values were measured at 6 rpm for primers and 10 rpm for adhesives, at a temperature of 25°C and room temperature of 24.5°C and humidity of 17.3-percent in a dark room. All materials were tested at the same time in an effort to eliminate temperature variations which can affect the viscosity readings.

PREPARATION OF DENTIN SPECIMENS

Sample size calculation was done based on previous studies [49-52] where the standard deviations of dentin permeability measurements were estimated to be around 15 percent. With a sample size of 10 specimens per group, this study had an 80-percent power to detect a difference of 20-percent between any two groups for dentin permeability, assuming two-sided tests conducted at a 5.0-percent significance level.

Sixty sound extracted human molars were collected. Teeth were cleaned of attached soft tissues and autoclaved in water for 20 minutes. Each tooth was used to make one 1 mm-thick dentin disc from dentin that was under the occlusal enamel and above the

pulp horns. To prepare teeth for sectioning, each tooth was mounted in a mounting acrylic resin (Lecoset® 7008, Leco Corporation, Saint Joseph, MI, USA) (Figure 10). Before mounting, teeth were marked with two lines, one marking the cemento-enamel junction (CEJ) and the other made occlusal to the CEJ mark where the cusps end at the junction of occlusal and middle thirds of the crown (Figure 10). Teeth were then mounted by embedding the roots up to the CEJ mark in the acrylic resin (Figure 10). During mounting, teeth were attached to a dental surveyor to hold the tooth in a stable position parallel to its long axis while the acrylic resin is setting (Figure 10). After that, the resin block with the mounted tooth was secured on a cutting machine (Isomet 1000 Precision Saw, Buehler, Lake Bluff, IL, USA) with a mounted diamond disk (Isomet Diamond Wafering Blades, Buehler, Lake Bluff, IL, USA) that was used at 200 rpm and under water cooling to make a cut along the second line mark in order to remove the occlusal third of the crown and expose dentin ensuring no enamel was left on the surface (Figure 11). Next, a manual milling machine (CNC Benchtop Mill, MicroKinetics Corporation, Kennesaw, GA, USA) with a mounted diamond hole saw with an external diameter of 9.89 mm (Neiko 00823A Diamond Grit Hole Saw Drill 3/8”, China) was used to remove the enamel on the periphery of the crown and create a dentin stump with a diameter of 7.20 mm (Figure 12). The resin block was then transferred back to the cutting machine with the diamond disk to cut a dentin disc at a thickness of about 1.2 mm (Figure 13). The last step was to finish both surfaces of the dentin discs using a polishing machine (Spectrum System 1000, Leco Corporation, Saint Joseph, MI, USA) with 1200-grit silicon carbide abrasive paper (1200-grit, Leco Corporation, Saint Joseph, MI, USA) (Figure 14) in order to obtain flat disc surfaces and a final thickness 1 mm (±0.1) (Figure

15). A bur was used to create an indentation on the occlusal surface for identification, because this was the surface to be treated (Figure 15). Discs were then individually stored in wells of a 96-well plate with absorbent paper (Kimtech wipes, Kimberly-Clark Professional, Fisher Scientific, USA) damped with deionized water to provide 100-percent humidity and stored at 4°C (±1) until use.

FIRST DENTIN PERMEABILITY MEASUREMENT (BASELINE)

Before starting dentin permeability measurements, dentin discs were immersed and sonicated in 17.0-percent ethylene diaminetetraacetic acid (EDTA) solution for 10 minutes to remove the smear layer and open the dentinal tubules simulating dentin in hypersensitive teeth and then in deionized water for another 10 minutes to remove residual EDTA and debris of smear layer. Effectiveness of 17.0-percent EDTA in removing the smear layer was verified by SEM to visualize opening of the dentinal tubules after use. One dentin disc was used, and half of the disc surface was covered with tape and then the disc was immersed and sonicated in 17.0-percent EDTA for 10 minutes; then the tape was removed, and the specimen was sonicated for another 10 minutes in deionized water. After that, the dentin disc was dried and sputter coated with gold in preparation for scanning under SEM (JSM-6390LV SEM, JEOL, Peabody, MA, USA) (Figure 16).

After that, discs were ready for the first, baseline, permeability measurement. Dentin permeability was tested by measuring the hydraulic conductance using a dentin permeability testing machine (Odeme® Dental Research, Luzerna, SC, Brazil) (Figure 17). Figure 18 is a schematic illustration of the dentin permeability measurement using this machine. The machine uses a water flow system under a simulated pulpal pressure of

5 psi. The dentin disc was stabilized between two O-shaped rubber rings with the occlusal side facing up and the pulpal side facing down, allowing water in the system to pass through the disc from the pulpal to the occlusal surface, simulating the outward movement of the dentinal fluid under the influence of pulpal pressure. Water from a water reservoir passed into a horizontal 100-μm microcapillary tube and then through the dentin disc. An air bubble within the water in the microcapillary tube was induced using the bubble injection device and its linear displacement was measured over 3 minutes. This measurement was repeated three times and the mean displacement value was used. Permeability was calculated according to the equation; Lp=Q/PA, where Lp is the dentin permeability (μl/min.cmH_2O.cm^2), Q is the filtration index (μl/min), P is the hydrostatic pressure applied (5 psi = 351.535 cmH_2O) and A is the exposed surface area of the dentin disc (0.05817 cm^2). Q is calculated using the equation; Q=VD/LT, where V is the volume of the microcapillary tube (100 μl), D is the displacement of the air bubble inside the microcapillary tube (mm), L is the length of the microcapillary tube (116 mm) and T is the test time (3 min). The permeability calculation was done using the software Analysis v4.0 (Odeme® Dental Research, Luzerna, SC, Brazil). This baseline permeability was considered the initial permeability for each dentin disc (%Lp_0 = 100%).

TREATMENT APPLICATION

Dentin discs were randomly allocated to one of six treatment groups (n=10), using a random sequence generator (Random.org) according to the treatment protocol (Table I); NC: negative control; SBMP: SBMP primer and SBMP adhesive; HNT-PR: arginine-$CaCO_3$-HNT primer and SBMP adhesive; HNT-ADH: SBMP primer and arginine-$CaCO_3$-HNT adhesive; HNT-PR+ADH: arginine-$CaCO_3$-HNT primer and arginine-

$CaCO_3$-HNT adhesive, and COL: Colgate Sensitive Pro-relief used as a prophylaxis paste (Colgate-Palmolive Company, New York, NY, USA). Treatments were applied according to the manufacturer's instructions (Table I). Figure 19 shows the materials used for treatment.

SECOND DENTIN PERMEABILITY MEASUREMENT (POST-TREATMENT)

After the dentin discs were treated, they were stored in 100-percent humidity at 4°C (±1) and 24 hours later, a second permeability measurement was carried out ($\%Lp_1$).

EROSION-ABRASION-REMINERALIZATION CYCLING

In order to simulate challenges in the oral environment, specimens were submitted to a sequence of erosive and abrasive challenges. A five-day erosion-abrasion-remineralization model was utilized.[53] Figure 20 illustrates a one-day cycle that was repeated for five days. Artificial saliva (pH ≅ 7.0) and 0.3-percent citric acid (pH ≅ 2.6) were prepared just before starting the cycling. Artificial saliva was prepared according to the Indiana University School of Dentistry Oral Health Research Institute (IUSD-OHRI) recipe (Table II). For citric acid, 15 g of the citric acid reagent was added to each 5 L of deionized water to get 0.3-percent citric acid.

For the erosive challenge, discs were immersed in 0.3-percent citric acid for 2 minutes and then rinsed with deionized water, four times a day. After each erosive challenge, discs were immersed in artificial saliva for 60 minutes. Discs were transferred from one solution to another in customized 24-well plates with perforations at the bottom of wells to allow solutions to reach discs inside the wells (Figure 21). For the abrasive challenge, discs were brushed twice a day, in the middle of the first and last

remineralization periods. This was performed using a custom-made automated brushing machine with Oral-B® Indicator Flat Trim Soft-bristle toothbrush heads (Figure 22). A toothpaste slurry was prepared with a 1:3 ratio of toothpaste (Colgate® Triple Action Toothpaste) to deionized water and used to brush specimens (Figure 23). It was kept on a stirring plate at 300 rpm when not in use (Figure 23). Slurry was prepared for each day of cycling. Cube-shaped silicone holders with an indentation on the top surface that corresponded to the exact shape and size of the dentin discs were prepared with condensation silicone (Henry Schein Lab Putty Condensation Silicone, HSI, Melville, NY, USA) and were mounted on the brushing machine to hold the dentin discs while being brushed (Figure 24). Discs were brushed under a force of 200 g for 15 seconds (45 brushing strokes) with a total contact time with the toothpaste slurry of 2 minutes (Figure 25). Overnight, discs were stored in artificial saliva on a shaker (Benchmark Orbi-Shaker™ XL, Benchmark Scientific, Edison, NJ, USA) at 140 rpm and room temperature (Figure 26).

THIRD DENTIN PERMEABILITY MEASUREMENT (POST-CYCLING)

A third and final permeability measurement was carried out after the five-day cycling was completed ($\%Lp_2$).

STATISTICAL ANALYSIS

Cytocompatibility and dentin permeability data were analyzed using two-way analysis of variance (ANOVA), with factors for group and concentration for cytocompatibility, and group and time for dentin permeability. All pair-wise comparisons

from ANOVA analysis were made using Fisher's Protected Least Significant Differences to control the overall significance level at 5.0 percent. Two-sample t-test was used to analyze the viscosity.

RESULTS

CYTOCOMPATIBILITY

ANOVA analyses showed that for both LDH and WST-1, the group difference was significant (both $p < 0.0001$) and the concentration difference was significant (both $p < 0.0001$). The interactions between group and concentration were also significant for LDH ($p = 0.0451$) and WST-1 ($p = 0.0035$).

LDH Assay

All groups showed the highest cytotoxicity with aliquot concentration of 100 percent. At this concentration, HNT-PR group showed significantly greater cytotoxicity compared to SBMP ($p = 0.0388$) and HNT-PR+ADH ($p = 0.0188$) groups but not to HNT-ADH group ($p = 0.5464$). At 100-percent aliquot concentration, HNT-PR group was the only experimental group that was significantly more cytotoxic to HGF cells than the SBMP control group. The two other experimental groups, HNT-ADH and HNT-PR+ADH, were not more cytotoxic than the SBMP group. Cytotoxicity data are shown in Table III and Figure 27.

WST-1 Assay

At aliquot concentration of 100 percent, HNT-ADH group showed the highest cell viability compared with the three other groups, SBMP ($p = 0.0002$), HNT-PR ($p = 0.0043$) and HNT-PR+ADH ($p = 0.0014$), which had no significant differences between them. At 100-percent aliquot concentration, HNT-ADH group resulted in significantly greater HGF cell viability compared with the SBMP control group, while the two other

experimental groups, HNT-PR and HNT-PR+ADH, were similar to the SBMP group. Cell viability data are shown in Table IV and Figure 28.

VISCOSITY

Results from the two-sample t-tests showed a significant difference between the viscosity of the experimental and control primers ($p = 0.0023$), as well as that of the experimental and control adhesives ($p = 0.0292$). Both the experimental primer and adhesive had significantly increased viscosity compared with their controls. Viscosity data are shown in Table V.

DENTIN PERMEABILITY

ANOVA analyses showed that the group difference was significant ($p < 0.0001$) and the interaction between group and time was significant ($p < 0.0001$).

Comparisons between post-treatment ($\%Lp_1$) and post-cycling permeability ($\%Lp_2$) within each group showed that the NC group had a significantly greater $\%Lp_1$ than $\%Lp_2$ ($p < 0.0001$). For COL group, $\%Lp_1$ was significantly smaller than $\%Lp_2$ ($p = 0.0402$). All other groups showed no significant differences between $\%Lp_1$ and $\%Lp_2$.

Comparisons of $\%Lp_1$ and $\%Lp_2$ between groups showed that for $\%Lp_1$, all groups had a significantly smaller permeability compared with NC group (all $p < 0.0001$). For $\%Lp_2$, SBMP, HNT-PR, HNT-ADH and HNT-PR+ADH groups had a significantly smaller permeability compared with COL group ($p = 0.0172$, $p = 0.0291$, $p = 0.0196$ and $p = 0.0398$, respectively) but not with the NC group ($p = 0.2985$). Dentin permeability data are shown in Table VI and Figure 29.

FIGURES AND TABLES

FIGURE 1. Parts of the preparation of experimental materials. a) 50 ml tube containing ethanol that surfaced on top of the arginine-$CaCO_3$-HNT powder after centrifuge. b) Arginine-$CaCO_3$-HNT powder being weighed on scale. c) Amber jar containing SBMP adhesive with arginine-$CaCO_3$-HNT powder added to it. d) Arginine-$CaCO_3$-HNT adhesive after mixing.

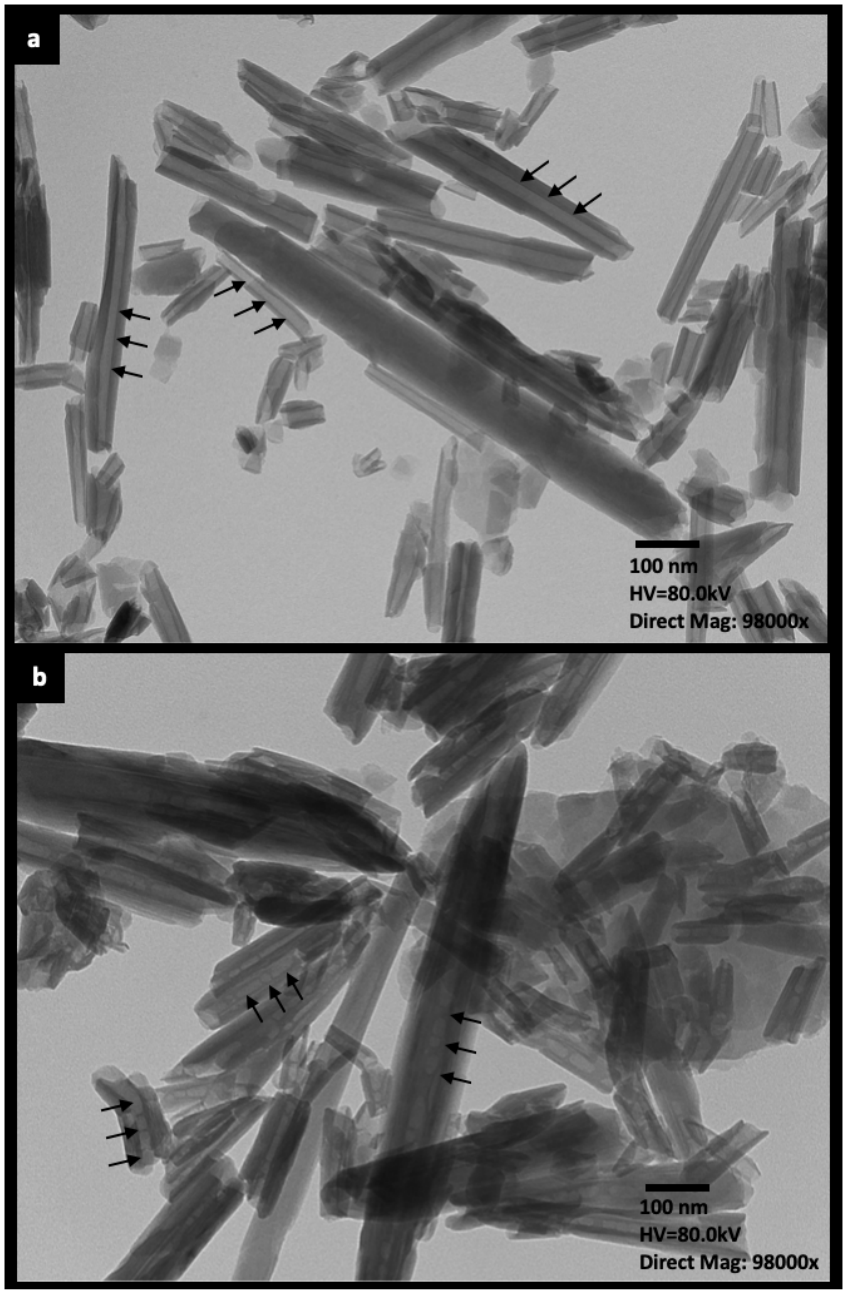

FIGURE 2(a) and 2(b). Transmission electron micrographs of HNT powder at X98000 magnification. a) Plain HNT powder before encapsulation. b) HNT powder after encapsulation with arginine and $CaCO_3$. Arrows are pointing at lumens of the nanotubes before and after encapsulation.

FIGURE 3. Prepared disc specimens of the four groups of different primer and adhesive combinations. From top to bottom rows; SBMP, HNT-PR, HNT-ADH and HNT-PR+ADH.

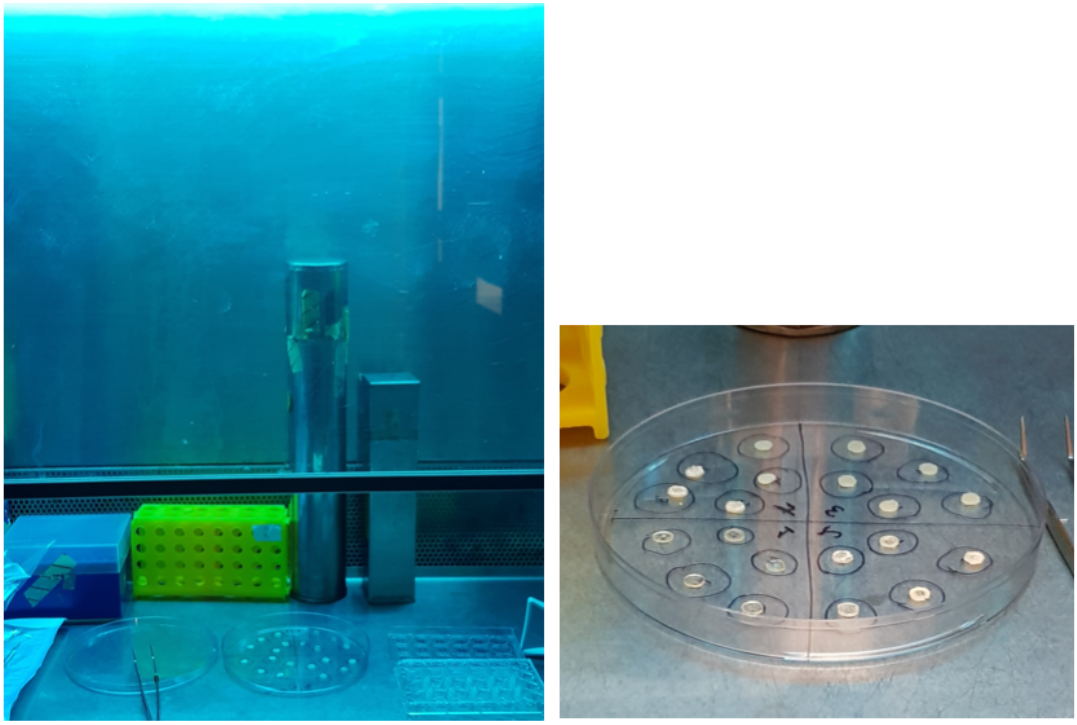

FIGURE 4. Discs being disinfected under UV light in a cell culture hood.

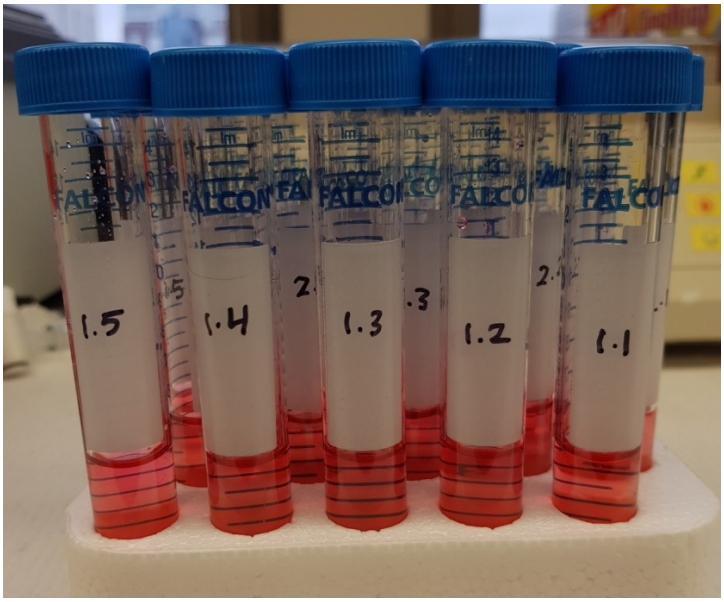

FIGURE 5. Discs individually incubated in DMEM in 15 ml tubes.

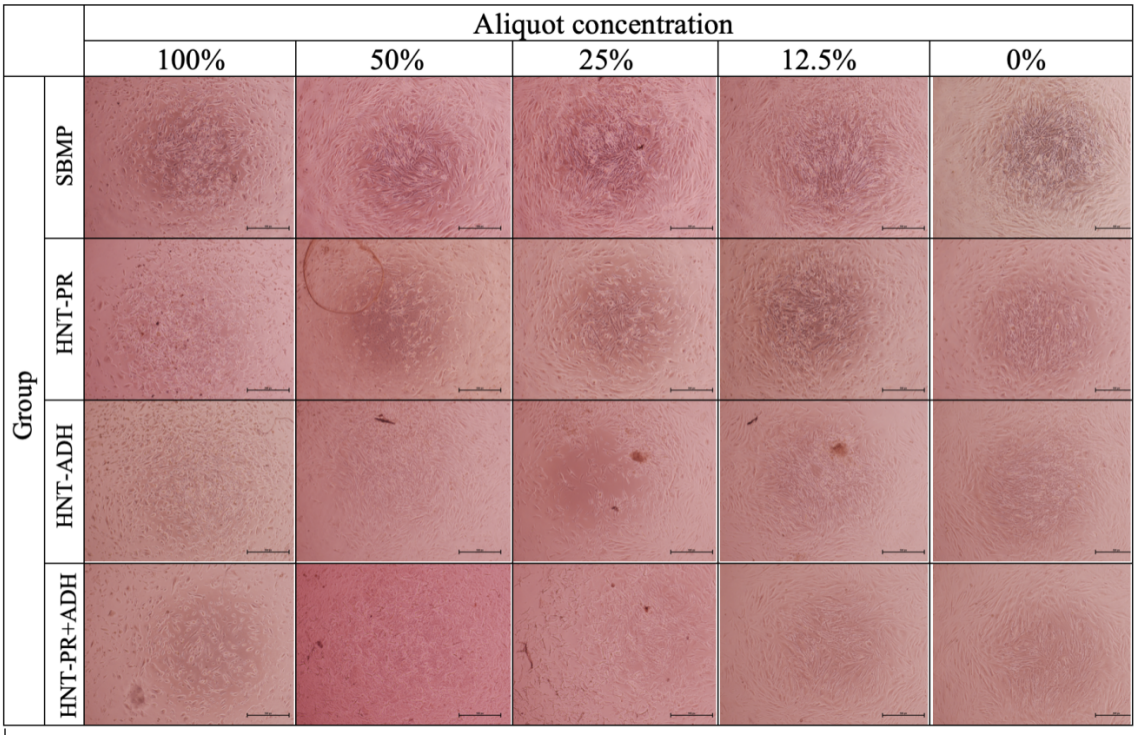

FIGURE 6. Light microscopy images of HGF cells after 24 hours of incubation with different concentrations of aliquots at 4× magnification.

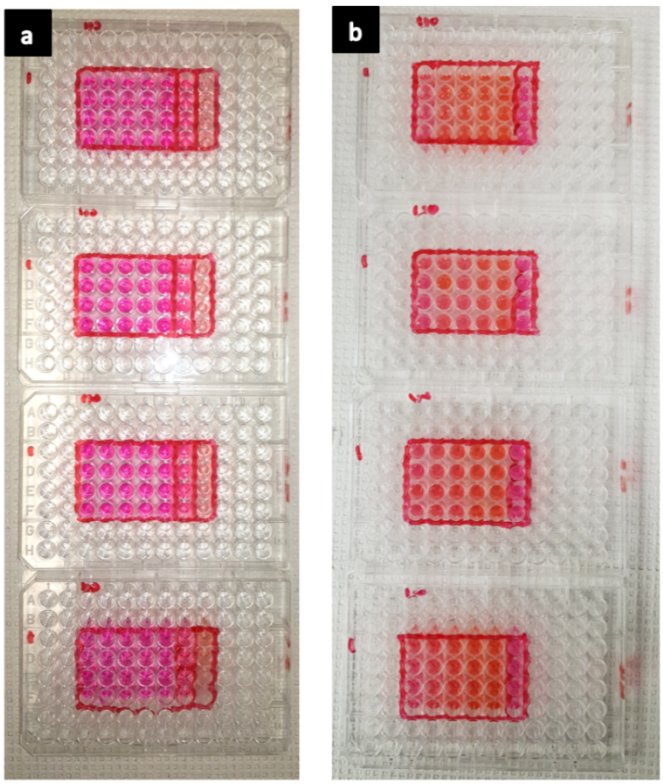

FIGURE 7. 96-well plates prepared for LDH (a), and WST-1 (b) assays and ready to go into the microplate reader.

FIGURE 8. Microplate reader (Molecular Devices).

FIGURE 9. Cup and Cone Viscometer. Test was performed under room temperature.

FIGURE 10. Tooth marking and mounting in acrylic resin with the use of a dental surveyor.

FIGURE 11. Removing the occlusal third of the crown using the cutting saw machine under water cooling.

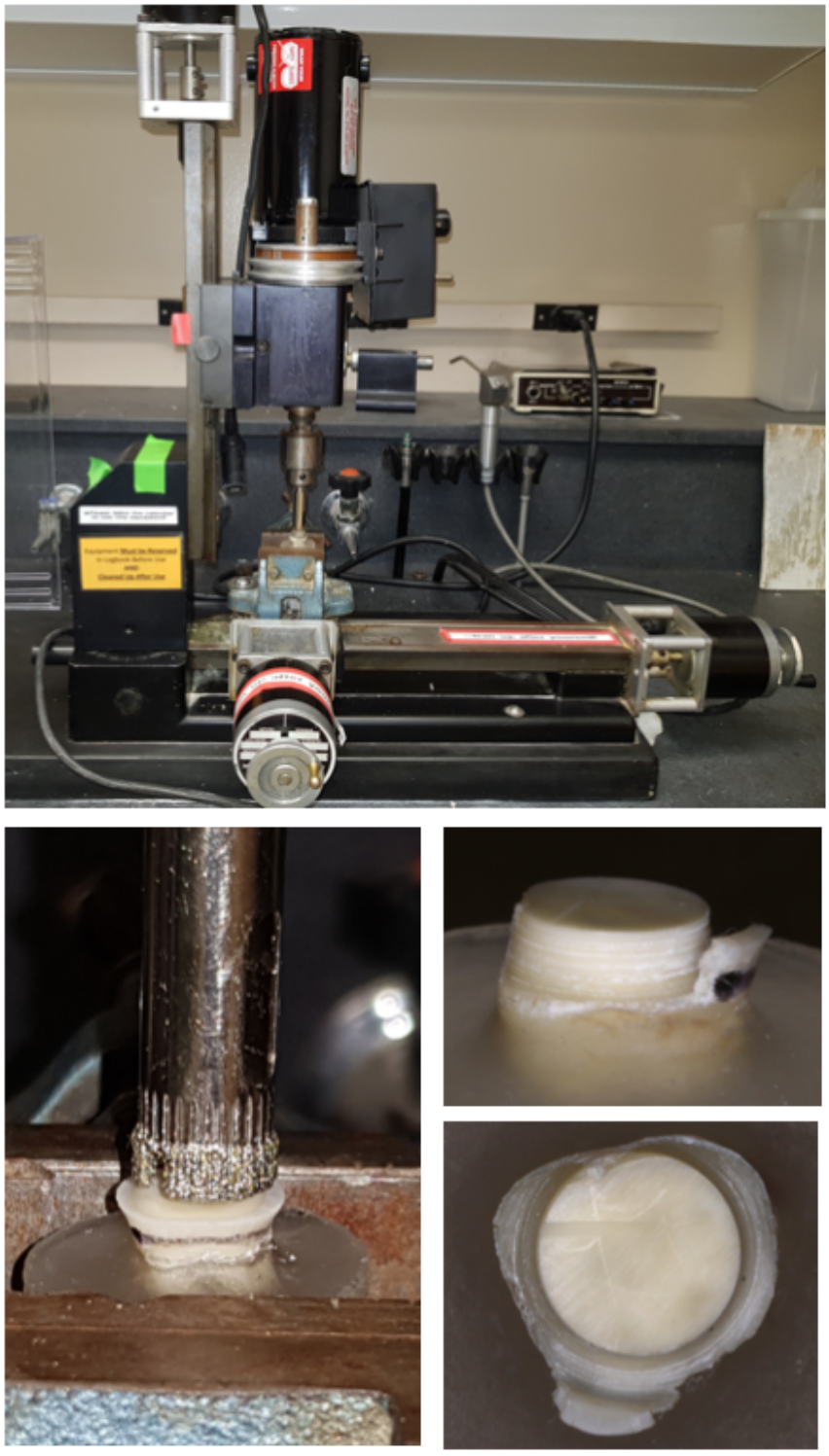

FIGURE 12. Removing the enamel on the periphery of the crown to create a dentin stump.

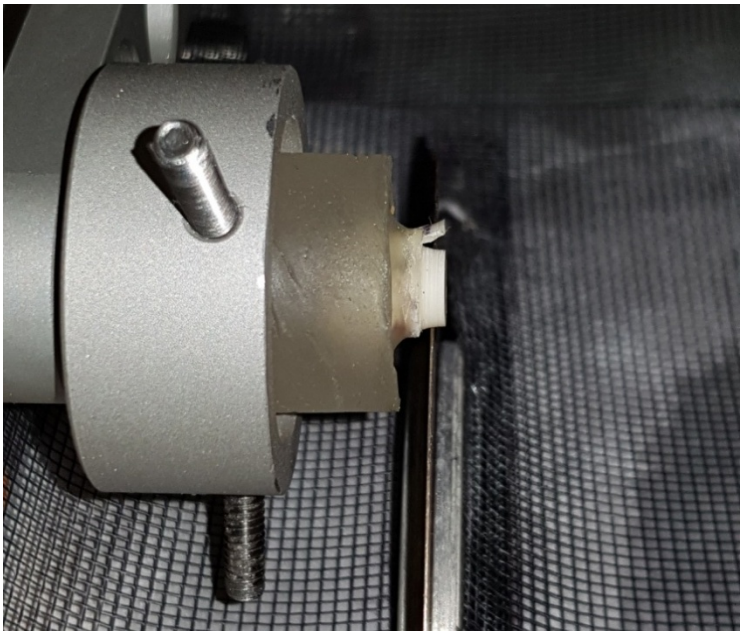

FIGURE 13. Cutting saw machine and diamond disk used to cut a 1.2 mm dentin disc.

FIGURE 14. Final polishing of the dentin disc using 1200 grit abrasive paper.

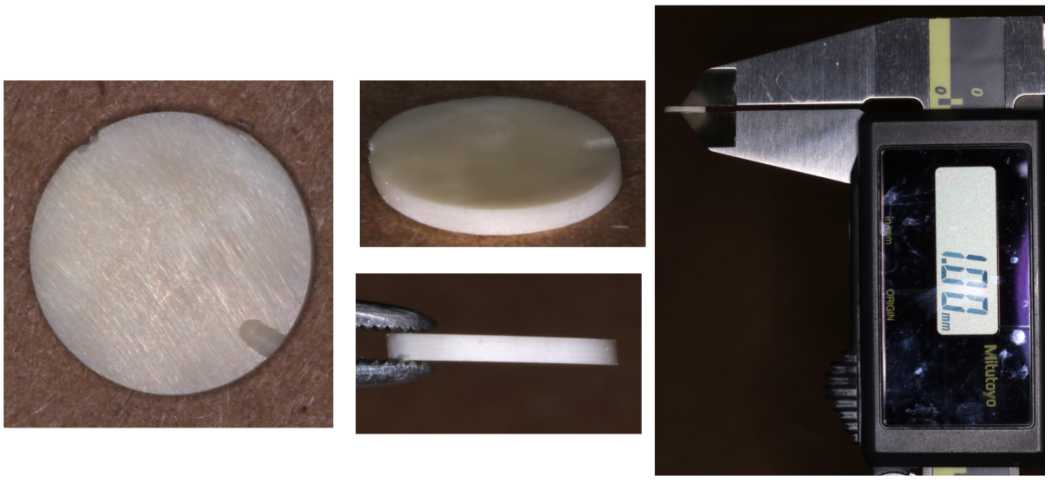

FIGURE 15. The final prepared dentin disc with 1±0.1 mm thickness and an indentation marking the occlusal surface.

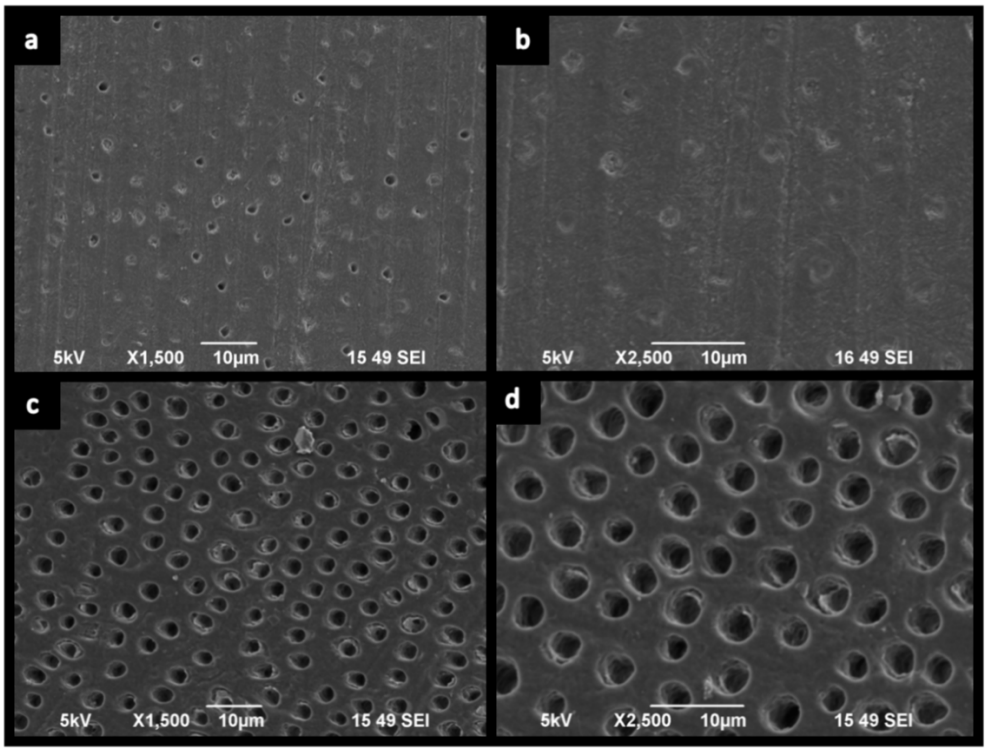

FIGURE 16. Scanning electron micrographs showing the effect of specimen immersion in 17% EDTA for 10 minutes. (a) and (b) show dentin that was not exposed to 17% EDTA at 1,500× and 2,500× magnification, respectively. (c) and (d) show dentin that was exposed to 17% EDTA at 1,500× and 2,500× magnification, respectively.

FIGURE 17. Dentin permeability testing machine (Odeme® Dental Research)

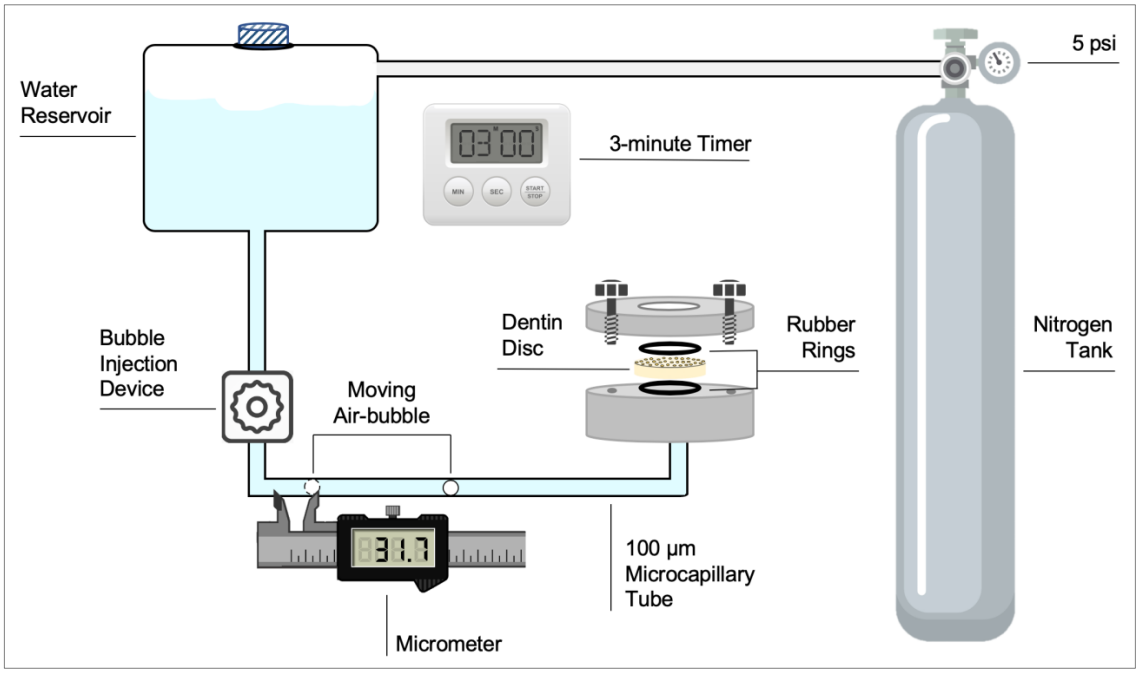

FIGURE 18. A schematic illustration of the dentin permeability measurement.

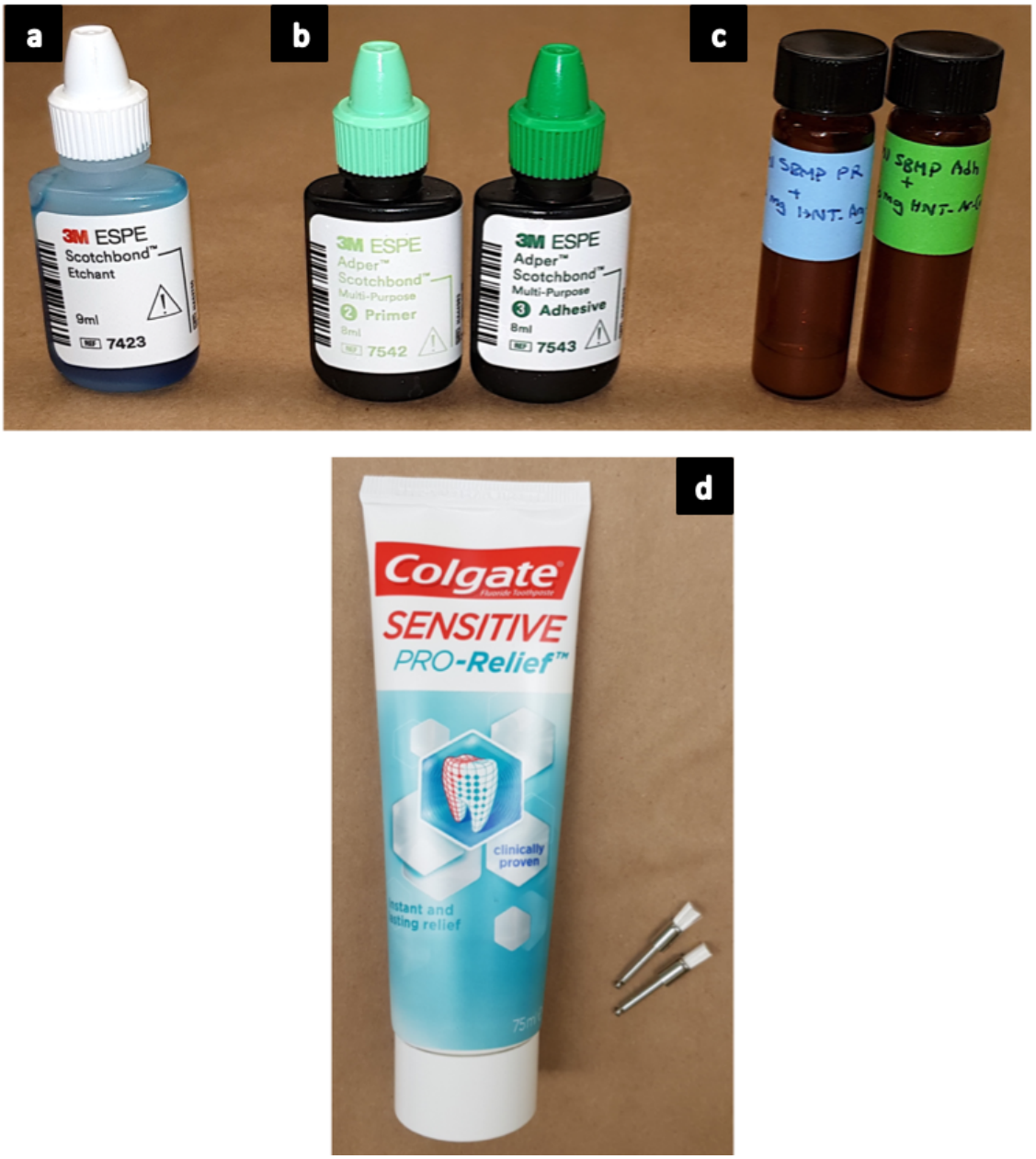

FIGURE 19. Materials used for treatment. a) SBMP etchant. b) SBMP primer and adhesive. c) Arginine-CaCO3-HNT modified primer and adhesive. d) Colgate® Sensitive Pro-relief ™ paste and prophy brushes.

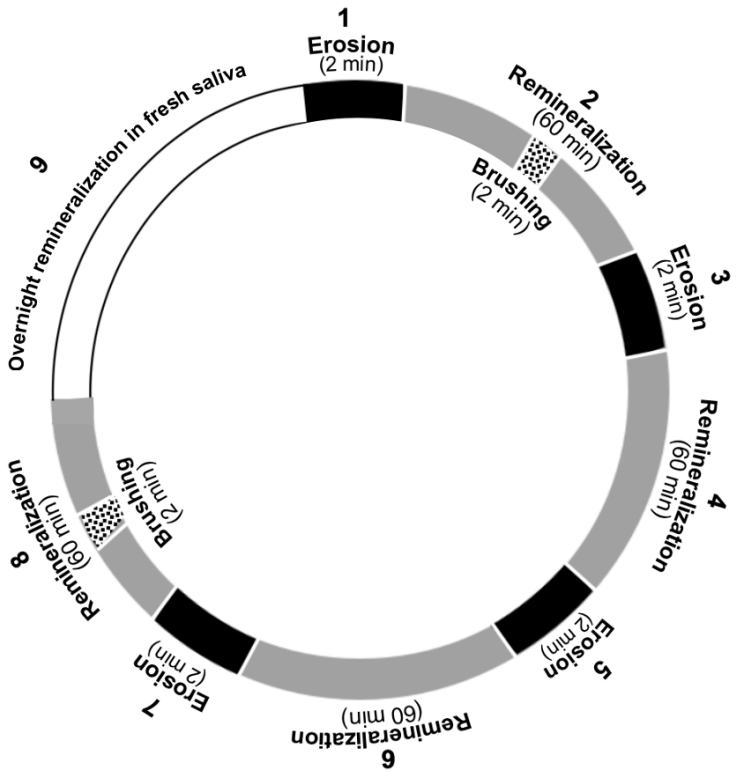

FIGURE 20. An illustration of one day of the five-day erosion-abrasion-remineralization cycling.

FIGURE 21. Specimens in customized perforated 24-well plates for transfer between different solutions.

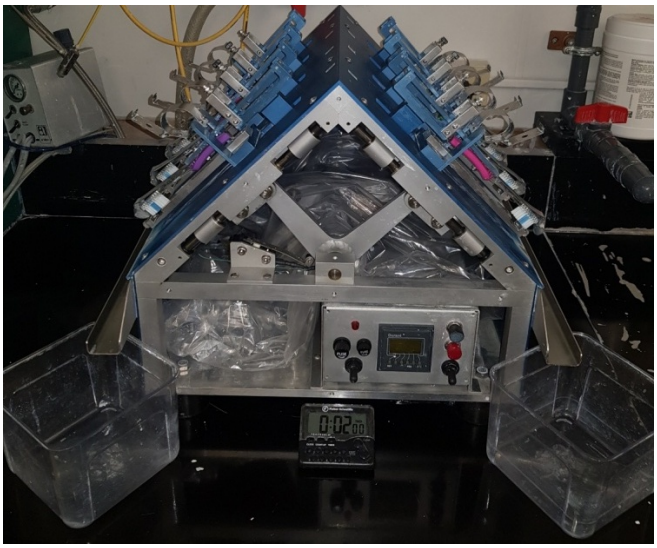

FIGURE 22. Automated brushing machine with mounted Oral-B® toothbrush heads.

FIGURE 23. Toothpaste slurry preparation.

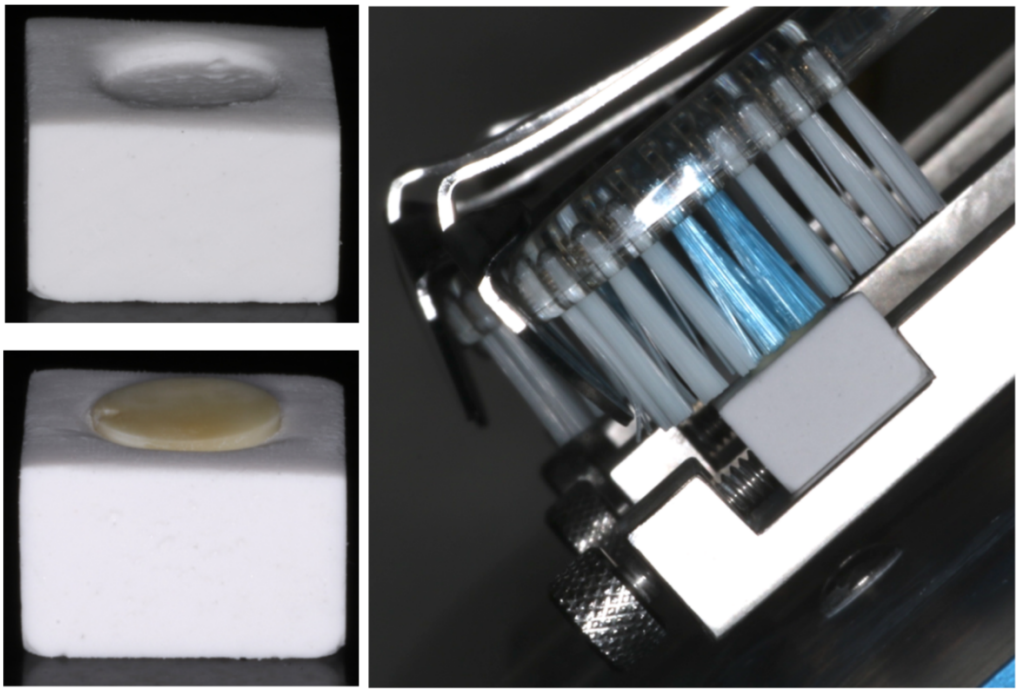

FIGURE 24. The prepared cube-shaped silicone holder mounted on the brushing machine and holding the dentin disc.

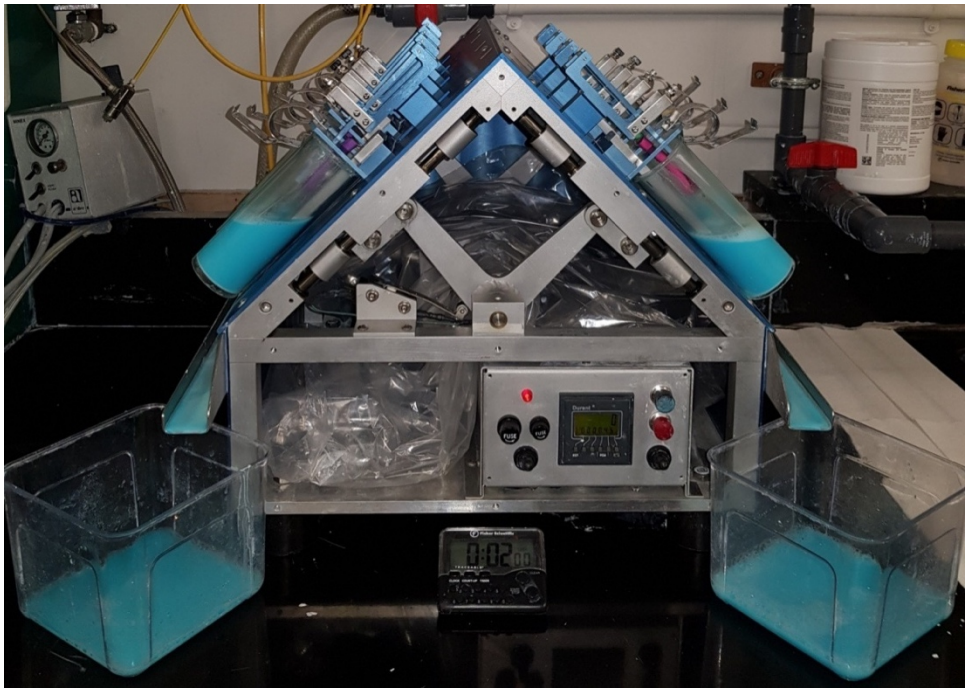

FIGURE 25. The brushing machine while specimens are being brushed.

FIGURE 26. Specimens stored in artificial saliva on a shaker overnight.

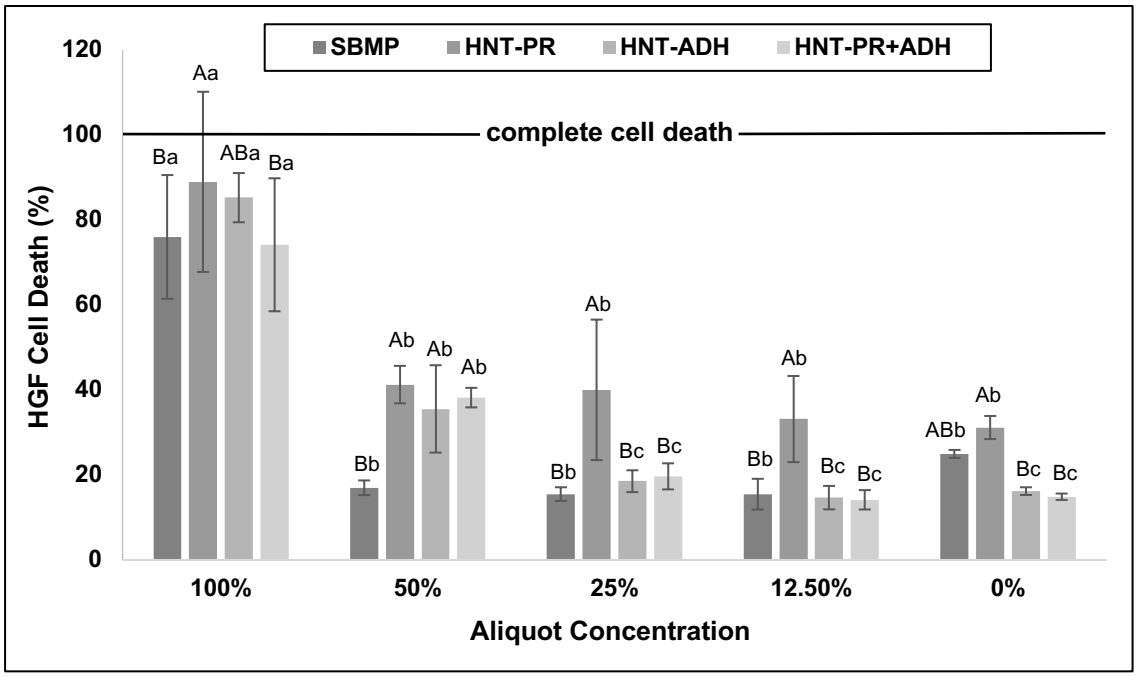

FIGURE 27. Cytotoxicity represented by HGF cell death (%) with different aliquot concentrations of the different groups. Different uppercase letters indicate statistically significant differences between different groups at the same concentration, different lowercase letters indicate statistically significant differences between different concentrations within the same group ($p<0.05$).

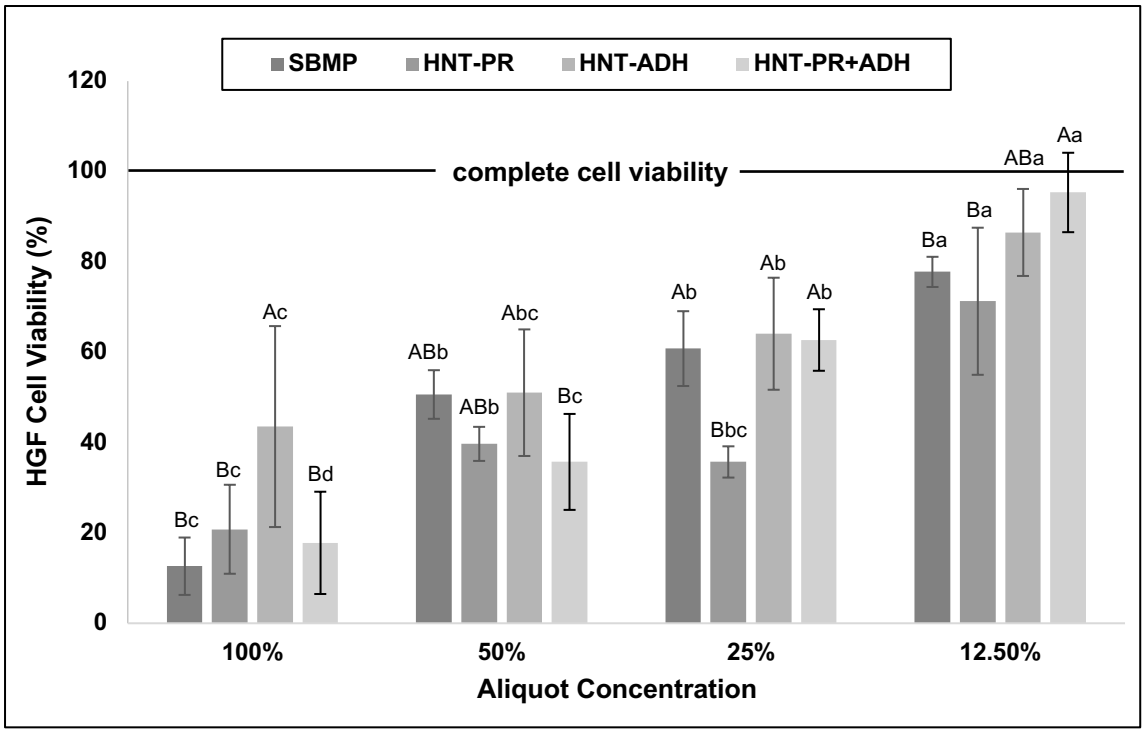

FIGURE 28. HGF cell viability (%) with different aliquot concentrations of the different groups. Different uppercase letters indicate statistically significant differences between different groups at the same concentration; different lowercase letters indicate statistically significant differences between different concentrations within the same group ($p < 0.05$).

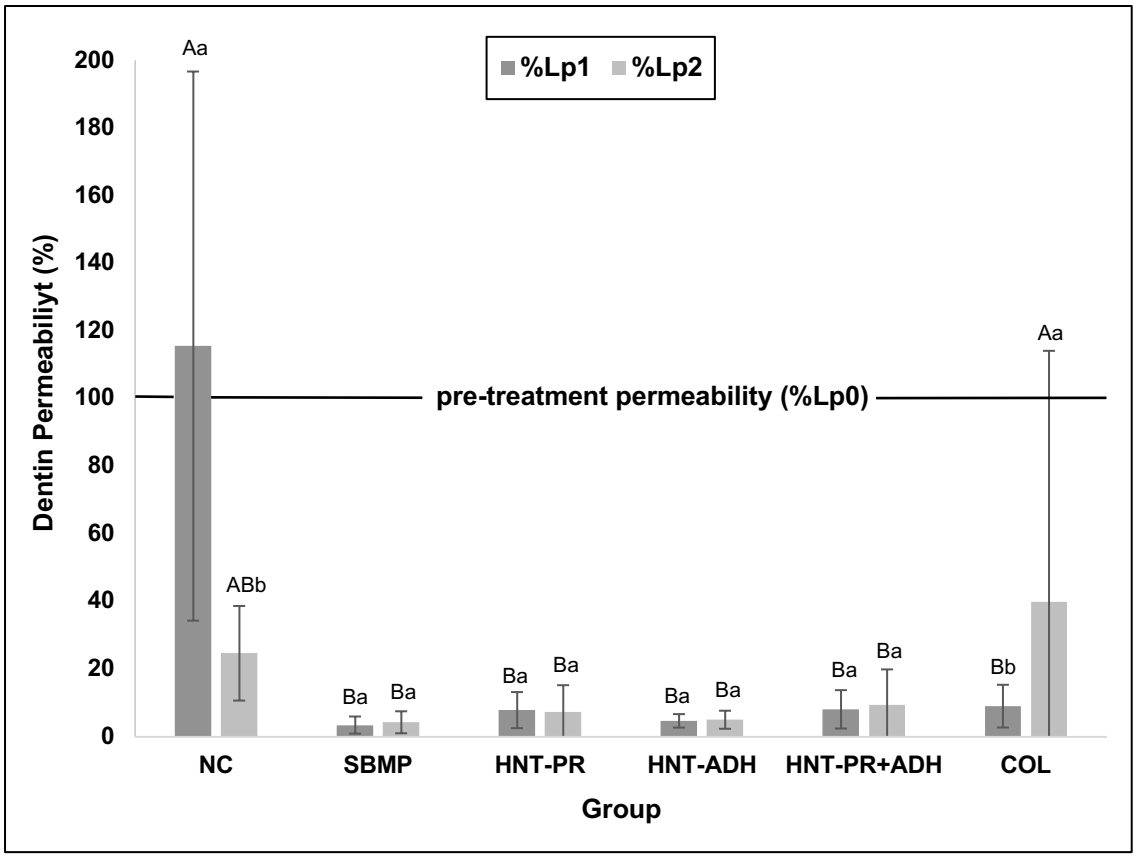

FIGURE 29. Dentin permeability (%) of all groups post-treatment ($\%Lp_1$) and post-cycling ($\%Lp_2$). Different uppercase letters indicate statistically significant differences between different groups for the same %Lp. Different lowercase letters indicate statistically significant differences between $\%Lp_1$ and $\%Lp_2$ within the same group ($p < 0.05$).

TABLE I

Treatment groups, materials compositions and application protocols

Treatment Group	Composition	Application protocol
NC (Negative control)	---	No treatment applied
SBMP (SBMP primer and adhesive)	**Etchant:** 35% phosphoric acid. **Primer:** water, HEMA, copolymer of acrylic and itaconic acids. **Adhesive:** Bis-GMA, HEMA, amines.	Etching (15 s), rinsing with deionized water (15 s), blot drying (Kimwipes), SBMP primer rubbed onto surface (5 s) then air-dried (5 s), SBMP adhesive applied and light cured for (10 s).
HNT-PR (Arginine-$CaCO_3$-HNT primer and SBMP adhesive)	**Etchant:** same as SBMP **Primer:** water, HEMA, copolymer of acrylic and itaconic acids, arginine-$CaCO_3$-encapsulated HNT. **Adhesive:** same as SBMP	Etching (15 s), rinsing with deionized water (15 s), blot drying (Kimwipes), arginine-$CaCO_3$-HNT primer rubbed onto surface (5 s) then air-dried (5 s), SBMP adhesive applied and light cured for (10 s).
HNT-ADH (SBMP primer and Arginine-$CaCO_3$-HNT adhesive)	**Etchant:** same as SBMP **Primer:** same as SBMP **Adhesive:** Bis-GMA, HEMA, amines, arginine-$CaCO_3$-encapsulated HNT.	Etching (15 s), rinsing with deionized water (15 s), blot drying (Kimwipes), SBMP primer rubbed onto surface (5 s), then air-dried (5 s), arginine-$CaCO_3$-HNT adhesive applied and light cured for (10 s).

(TABLE I continued)

TABLE I (cont.)

Treatment groups, materials compositions and application protocols

HNT-PR+ADH (Arginine-$CaCO_3$-HNT primer and arginine-$CaCO_3$-HNT adhesive)	**Etchant:** same as SBMP **Primer:** water, HEMA, copolymer of acrylic and itaconic acids, arginine-$CaCO_3$-encapsulated HNT. **Adhesive:** Bis-GMA, HEMA, amines, arginine-$CaCO_3$-encapsulated HNT.	Etching (15 s), rinsing with deionized water (15 s), blot drying (Kimwipes), arginine-$CaCO_3$-HNT primer rubbed onto surface (5 s) then air-dried (5 s), arginine-$CaCO_3$-HNT adhesive applied and light cured for (10 s).
COL (Colgate® Sensitive Pro-relief ™ paste)	Arginine, hydrated silica, calcium carbonate, glycerin, water, bicarbonate, sodium saccharine.	With a rotary prophylaxis brush used in a low-speed handpiece, paste is applied to dentin for 3 s, then repeated for a total of 1 min.

TABLE II

IUSD-OHRI recipe for preparing artificial saliva

To make 5 L of artificial saliva, dissolve the ingredients below in 5 L of deionized water:	
$CaCl_2*H_2O$	1.065 g
KH_2PO_4	3.69 g
KCl	5.57 g
NaCl	1.905 g
Tris Buffer	60 g
Gastric Mucin	11 g

TABLE III

Cytotoxicity (%) as calculated from the LDH assay; mean (SD)

	Aliquot Concentration				
Group	**100%**	**50%**	**25%**	**12.5%**	**0%**
SBMP	76.03 (14.56) ^{Ba}	16.99 (1.73) ^{Bb}	15.51 (1.60) ^{Bb}	15.48 (3.62) ^{Bb}	24.96 (0.94) ^{ABb}
HNT-PR	88.99 (21.20) ^{Aa}	41.27 (4.41) ^{Ab}	40.04 (16.53) ^{Ab}	33.16 (10.14) ^{Ab}	31.17 (2.72) ^{Ab}
HNT-ADH	85.27 (5.79) ^{ABa}	35.55 (10.28) ^{Ab}	18.54 (2.57) ^{Bc}	14.67 (2.76) ^{Bc}	16.20 (0.91) ^{Bc}
HNT-PR+ADH	74.18 (15.65) ^{Ba}	38.21 (2.30) ^{Ab}	19.68 (3.07) ^{Bc}	14.16 (2.28) ^{Bc}	14.88 (0.75) ^{Bc}

Different uppercase letters indicate statistically significant differences between different groups at the same concentration (vertical); different lowercase letters indicate statistically significant differences between different concentrations within the same group (horizontal) ($p < 0.05$).

TABLE IV

Cell viability (%) as calculated from the WST-1 assay; mean (SD)

	Aliquot Concentration			
Group	**100%**	**50%**	**25%**	**12.5%**
SBMP	12.63 (6.35) ^{Bc}	50.64 (5.38) ^{ABb}	60.80 (8.28) ^{Ab}	77.78 (3.34) ^{Ba}
HNT-PR	20.81 (9.85) ^{Bc}	39.71 (3.77) ^{ABb}	35.71 (3.46) ^{Bbc}	71.28 (16.28) ^{Ba}
HNT-ADH	43.54 (22.24) ^{Ac}	51.04 (14.01) ^{Abc}	64.09 (12.40) ^{Ab}	86.51 (9.63) ^{ABa}
HNT-PR+ADH	17.80 (11.32) ^{Bd}	35.72 (10.62) ^{Bc}	62.70 (6.81) ^{Ab}	95.36 (8.79) ^{Aa}

Different uppercase letters indicate statistically significant differences between different groups at the same concentration (vertical); different lowercase letters indicate statistically significant differences between different concentrations within the same group (horizontal) ($p < 0.05$).

TABLE V

Viscosity (cP) of control (SBMP) and experimental (Arginine-$CaCO_3$-HNT) primers and adhesives; mean (SD)

	Primers	**Adhesives**
SBMP	9.3 (0) ^{b}	437.7 (2.8) ^{B}
Arginine-$CaCO_3$-HNT	25.8 (5.0) ^{a}	580.6 (13.9) ^{A}

Different lowercase letters indicate statistically significant differences between different primers; different uppercase letters indicate statistically significant differences between different adhesives ($p < 0.05$).

TABLE VI

Dentin permeability (%), pre-treatment ($\%Lp_0$), post-treatment ($\%Lp_1$) and post-cycling ($\%Lp_2$); mean (SD)

Group	**$\%Lp_0$**	**$\%Lp_1$**	**$\%Lp_2$**
NC	100	115.50 (81.14) ^{Aa}	24.71 (13.98) ^{ABb}
SBMP	100	3.50 (2.53) ^{Ba}	4.32 (3.25) ^{Ba}
HNT-PR	100	7.92 (5.33) ^{Ba}	7.44 (7.84) ^{Ba}
HNT-ADH	100	4.74 (1.99) ^{Ba}	5.07 (2.67) ^{Ba}
HNT-PR+ADH	100	8.15 (5.67) ^{Ba}	9.40 (10.55) ^{Ba}
COL	100	9.09 (6.31) ^{Bb}	39.91 (74.19) ^{Aa}

Different uppercase letters indicate statistically significant differences between different groups (vertical); different lowercase letters indicate statistically significant differences between $\%Lp_1$ and $\%Lp_2$ within the same group (horizontal) ($p < 0.05$).

DISCUSSION

Most dentin desensitizing agents are effective at first, but over time, the material occluding the dentinal tubules will dissolve, leading to re-opening of the tubules and recurrence of pain. The goal of the present study was to attempt to develop an agent that would mechanically occlude the tubules for extended periods of time and also carry components that would favor natural tooth mineralization. For this purpose, an adhesive system was used as a vehicle to carry arginine to help to mitigate an acidic environment, and to carry calcium carbonate as a source of calcium.

Based on previous research, encapsulating active components such as doxycycline and chlorhexidine into HNTs and incorporating up to 20 wt % of these encapsulated HNTs into adhesive systems was shown to be promising with no detrimental effects on basic material properties, such as cytocompatibility, degree of conversion, microhardness and water sorption.[23, 42, 44-46, 48] Based on this, in the present study, the primer and adhesive of a commercial adhesive system were modified with 15 wt% of the arginine-$CaCO_3$-HNT powder. The Adper ™ Scotchbond ™ Multi-Purpose Plus (SBMP) adhesive system was used to be able to make comparisons with other studies that have used the same system for modification with nanotubes.

The HNT powder was examined under transmission electron microscopy (TEM) before and after encapsulation with arginine and calcium carbonate (Figure 2). As demonstrated in the TEM images, the hollow lumens of the HNTs appear to be empty in the plain HNT powder (Figure 2a). However, in the encapsulated HNT powder (Figure 2b), the HNT lumen are filled, which could indicate successful encapsulation. Further

investigation can confirm the presence of arginine and $CaCO_3$. Agglomerates of HNTs can be seen in Figure 2 (a and b) as a result of specimen preparation for the TEM imaging, which involves mixing the nanotube powder with water. When incorporating fillers, in this case the nanotubes, into a resin matrix, it is important to ensure homogenous dispersion. For this purpose, in the present study, the primer and adhesive were mixed with the encapsulated HNTs using a mechanical mixer, which can break agglomerates that may form.[54]

The null hypothesis that there will be no difference between the modified and non-modified adhesive systems regarding cytocompatibility was rejected as the modified adhesive group showed higher cell viability compared with all other groups at a 100-percent aliquot concentration. The only experimental group that showed a negative effect on the HGF cells was the modified primer group. However, this negative effect was only visible in the cytotoxicity results from the LDH assay but was not corroborated by decreased cell viability from the results of the WST-1 assay. Additionally, the modified primer was also used in the modified primer and adhesive group without showing an increased cytotoxicity or decreased cell viability at the 100-percent aliquot concentration. For these reasons, that finding should be interpreted with caution. Results from the WST-1 assay demonstrate that at 100-percent aliquot concentrations, all three experimental groups showed an equal or greater HGF cell viability compared with the non-modified adhesive group.

Viscosity of dental adhesives greatly influences their ability to penetrate into the dentinal interfibrillar spaces to form a reliable hybrid layer.[55] In the present study, the null hypothesis regarding viscosity was rejected as both the experimental primer and

adhesive demonstrated higher viscosity than the control primer and adhesive. The viscosity values that were obtained by our testing for the control primer (9.3 cP) and adhesive (437.7 cP) were very close to the values reported by the materials' manufacturer (10.7 cP for the primer and 355 cP to 455 cP for the adhesive).[56, 57] This confirms the validity of the testing conditions that were followed in the present study. From observation of the modified primer and adhesive during application to dentin, only a slight increase in both materials' viscosities was noted, which did not make handling or application more difficult. A different study evaluated the viscosity of primer and adhesive modified with nanotubes encapsulated with chlorhexidine and showed that although the addition of nanotubes significantly increased the viscosity, the bond strength to dentin was not affected.[48] Therefore, the increased viscosity of the modified primer and adhesive in the present study is not thought to have negatively impacted their bonding performance.

EDTA is a chelating agent commonly used in endodontics to remove the smear layer via binding to inorganic substrates such as calcium.[58,59] The present study demonstrated that immersing dentin specimens in 17-percent EDTA for 10 minutes can successfully remove the smear layer and plugs to open the dentinal tubules to simulate hypersensitive dentin, as evident in the scanning electron micrographs (Figure 16).

Results from the dentin permeability test show wide variations. Other laboratory dentin permeability studies have reported the same observation.[60, 61] This is likely a result of the dentin discs being prepared from teeth extracted from individuals of variable ages and also variations in the depth from which the dentin discs were obtained, which consequently can result in variable permeabilities.

In regard to post-treatment dentin permeability ($\%Lp_1$), all groups except for the negative control group showed a reduced permeability compared with their baseline permeability. No significant differences were found between the groups and all of them were significantly lower than the negative control group. This was not unexpected, since all interventional groups were expected to show a drop of permeability after treatment. These results indicate that the use of adhesive systems, modified or not, and the use of the prophy paste both provided an immediate occlusion of the tubules to reduce permeability.

The post-cycling permeability data ($\%Lp_2$) reflect the tubule occlusion after being challenged by erosive and abrasive challenges simulating those present intraorally. All experimental groups maintained their reduced post-treatment permeability with no differences between their $\%Lp_2$ and $\%Lp_1$. The prophy paste group showed an increased $\%Lp_2$ compared with its $\%Lp_1$, as well as compared with $\%Lp_2$ of all adhesive groups. The negative control group showed a significant drop of permeability after cycling, with no significant differences compared with any of the other groups. Based on this, the null hypothesis stating no difference between the experimental and control groups in the ability to reduce dentin permeability was rejected.

The negative control group showed an increased post-treatment permeability that exceeded baseline permeability. Fluctuations in dentin permeability as an effect of storage medium and duration have been reported in previous studies.[60, 62] A study has shown an increase in the permeability of a group that received no treatment from 100 percent at baseline to 103.74 percent after one day and 128.62 percent after 90 days of storage in deionized water.[62] In general, there seems to be a tendency for water and water-based storage media to increase dentin permeability due to washout of residual

intratubular contents by the water.[60] To minimize this water-washout effect, specimens in the present study were not stored by immersion in water, but instead were stored in humidity created by wet absorbent paper, without water pooling.

Regarding the post-cycling permeability of the negative control group, some decrease in dentin permeability was expected because the specimens were brushed with a toothpaste containing sodium fluoride along with other abrasives. These specimens were immersed in artificial saliva saturated with mineral salts, which could cause tubule occlusion. A decrease in dentin permeability from 100 percent at baseline to 30 percent was previously reported as a result of brushing with distilled water only.[51] Results from a different study also showed permeability reduction from 100 percent to 88 percent when brushing with distilled water.[52] This reduction in permeability after toothbrush simulation is attributed to the formation of smear layer by the brushing action, which can block the dentinal tubules.[51]

Besides brushing, artificial saliva has dual opposite actions: 1) Increasing permeability by solubilizing any precipitating particles blocking the tubules, and 2) Reducing permeability by providing a source of minerals that could precipitate inside the tubules.[51] Studies have reported variable effects of immersion in artificial saliva on dentin permeability. The total amount of time for which specimens are immersed inside the artificial saliva is the main factor that dictates which action would predominate. In the present study, specimens were immersed in artificial saliva for a total of about 118 hours during the five-day cycling period, and because of this long time, our results of decreased post-cycling permeability reflect a mineral precipitation action exceeding the solubilization action. A study has shown an increase in permeability after immersion in

artificial saliva for 24 hours,[51] while another study reported a reduced permeability after the same immersion time,[52] possibly due to variations in the compositions of the artificial saliva.

For the prophy paste group, the permeability was significantly reduced to 9.09 percent after treatment, but then increased to 39.91 percent after cycling and was not significantly different from the negative control group. A recent study evaluated dentin permeability of specimens treated with the same paste containing arginine and calcium carbonate and showed that the permeability dropped to 40 percent after treatment and then increased to about 65 percent after cycling.[63] The difference between their post-treatment and post-cycling permeabilities (25 percent) is close to the difference shown by our results (30.82 percent); however, their post-treatment permeability (40 percent) was much higher than ours (9.09 percent). This might be explained by the shorter duration of application that they used, which was 15 seconds, and the dilution of the paste with saliva compared with the present study where the paste was applied for one minute in full concentration. Although the manufacturer does not specify the required in-office application time, the present study aimed to mimic the clinical situation and provided an intermittent application of 3 seconds at a time, which the manufacturer recommends to prevent heat generation, for a total of one minute.

For groups that were treated with adhesive systems, all of them were able to reduce permeability with no differences between the modified and non-modified adhesives in the immediate post-treatment permeability. A point of interest was to evaluate if there would be any differences in the post-cycling permeability of these groups that could indicate differences in erosion and/or abrasion resistance. All four

groups had similar post-cycling permeabilities that were not different from their post-treatment permeabilities, meaning they maintained the reduced post-treatment permeability even after cycling. It is possible that a more aggressive cycling model than the one used may be able to show differences in erosion and/or abrasion resistance between the groups treated with the modified adhesive systems and the one treated with the non-modified system. Another limitation of the present study is that it did not reproduce some biological aspects of the oral environment, such as the presence of oral bacteria, essential to the mode of action of arginine in raising the pH, resulting in the inability to fully test the effect of arginine.

In conclusion, modification of the primer and adhesive did not negatively affect their cytocompatibility or ability to reduce dentin permeability after the abrasion-erosion-remineralization cycling. Specimens treated with the modified adhesive systems and with the commercial system presented significantly lower permeability after cycling compared with the ones treated with the prophy paste containing arginine and calcium carbonate. Further laboratory and clinical testing are required to demonstrate the potential benefits of the modified adhesive system.

SUMMARY AND CONCLUSIONS

The objective of this study was to modify the primer and adhesive of a commercial three-step etch-and-rinse adhesive system with 15 wt.% arginine-$CaCO_3$-encapsulated HNTs to be used for treatment of dentin hypersensitivity. Cytocompatibility, viscosity and ability to reduce dentin permeability were tested.

The null hypothesis stating no difference between the modified and non-modified adhesive systems regarding cytocompatibility and viscosity was rejected. In fact, both the experimental primer and adhesive demonstrated greater viscosity than the control primer and adhesive, and the modified adhesive group showed greater cell viability compared with all other groups at a 100-percent aliquot concentration.

The null hypothesis stating no difference between the experimental and control groups in the ability to reduce dentin permeability was also rejected. All three experimental groups showed significantly smaller values for post-cycling permeability compared with the prophy paste group.

The modification of the primer and adhesive did not negatively affect their cytocompatibility or ability to reduce dentin permeability after cycling. Dentin treated with the adhesive systems, with or without modification, showed significantly smaller values for post-cycling permeability compared with dentin treated with the prophy paste. The modified adhesive system needs to show the ability to release arginine and calcium, through additional research, to achieve the proposed clinical benefits.

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ABSTRACT

A MODIFIED ADHESIVE SYSTEM FOR USE IN TREATMENT OF DENTIN HYPERSENSITIVITY

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Background: Despite the variety of available treatments for dentin hypersensitivity (DH), it still can be challenging and unpredictable. Acidity in the oral environment poses a major challenge against a long-lasting occlusion of the dentinal tubules, whether achieved through applied agents or natural tooth mineralization. Arginine is effective in reducing the surrounding acidity, and when combined with the availability of calcium, can provide a favorable environment for tooth mineralization.

Objectives: To modify the dentin primer and adhesive of a commercial adhesive system with arginine and calcium carbonate-encapsulated halloysite clay nanotubes (HNTs) and to evaluate their cytocompatibility, viscosity and efficacy in reducing dentin permeability for use as a treatment for DH.

Materials and Methods: Arginine and calcium carbonate were encapsulated into HNTs and the resulting powder was incorporated into the primer and the adhesive of a commercial three-step etch-and-rinse adhesive system (Adper ™ Scotchbond ™ Multi-Purpose Plus (SBMP)) at 15 wt%. To evaluate cytocompatibility, discs were prepared from four groups of materials; SBMP (non-modified primer and adhesive), HNT-PR (modified primer and non-modified adhesive), HNT-ADH (non-modified primer and modified adhesive) and HNT-PR+ADH (modified primer and adhesive). Human gingival fibroblast (HGF) cells were exposed to aliquots extracted from these discs and LDH and WST-1 assays were conducted to evaluate cell death and viability, respectively. A cup-and-cone viscometer was used to test the viscosity of the modified and non- modified primer and adhesive. To evaluate dentin permeability, 60 dentin discs were prepared and assigned to one of six treatment groups (n = 10); NC (negative control with no treatment); SBMP; HNT- PR; HNT-ADH; HNT-PR+ADH, and COL (Colgate® Sensitive Pro-relief ™ prophy paste). Dentin permeability was tested at three points: before treatment, 24 hours after treatment and after a five-day erosion-abrasion-remineralization cycling.

Results: For cytocompatibility, at full aliquot concentration, the LDH assay data showed that the HNT- PR group resulted in a significantly higher cytotoxicity compared with SBMP and HNT-PR+ADH groups, but not with HNT-ADH group. The WST-1

assay data showed that the HNT-ADH group resulted in the highest cell viability compared with the three other groups. For viscosity, both the modified primer and adhesive showed a significantly increased viscosity compared with the non-modified primer and adhesive. For post-treatment dentin permeability, all treatment groups showed a significantly lower permeability compared with the NC group. For post-cycling permeability, SBMP, HNT-PR, HNT-ADH and HNT- PR+ADH groups showed a significantly lower permeability compared with COL group.

Conclusion: Modification of the primer and adhesive with arginine and calcium carbonate-encapsulated HNTs did not negatively affect their cytocompatibility or ability to reduce dentin permeability after the abrasion-erosion-remineralization cycling. Dentin treated with the adhesive systems, with or without modification, showed a significantly lower post-cycling permeability compared with dentin treated with the prophy paste.

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